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Characterization of a Hypothetical Protein Critical for the Symbiotic Interaction of *Aeromonas veronii* and *Hirudo verbana*

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Characterization of a Hypothetical Protein Critical
for the Symbiotic Interaction of
Aeromonas veronii and *Hirudo verbana*

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Honors Thesis

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I. ABSTRACT

The digestive tract symbiosis of the medicinal leech, *Hirudo verbana*, is a model system for studying the genes required for microbial colonization of digestive tracts, as *H. verbana* has only two species of bacteria that dominate the crop microbiota,

Aeromonas veronii and a *Rikenella*-like bacterium. Signature-tagged mutagenesis (STM) of the *A. veronii* strain, HM21R, revealed genes required for the colonization of the digestive tract. One of these mutants, JG573, has an interrupted gene that is predicted to encode a hypothetical protein. The region flanking the transposon insertion of this mutant was sequenced by primer walking. Comparison of the flanking DNA to the databases revealed similarity to the *rhs* gene, whose function is not known. PCR was performed to confirm the presence of a transposon insertion by detecting a size difference between JG573 and the parent strain. The wild type *rhs* gene was isolated from the fosmid library DNA, and a Southern blot was performed on fosmids obtained from the wild type and mutant DNA. To identify *in vitro* phenotypes of the mutant, the following experiments were performed: an SDS sensitivity assay, a growth curve, and a screen for two hundred-forty possible phenotypes using Biolog plates. The ability of the mutant to colonize the leech digestive tract was assessed in a competition assay, which revealed that this mutant had no colonization defect at 18 h after feeding but had a dramatic defect by 24 h. These data suggest that an important physiological change occurs during this time that affects the viability of this mutant inside the leech.

II. INTRODUCTION

Microbiota perform many important functions in the digestive tracts of animals. Often a diverse community of microbes colonize the digestive tract. For example, the human gut alone contains thousands of microbial species (Mahowald *et al.*, 2009). In order to discover genes required for digestive-tract colonization, microbe-host interactions of the gut need to be explored. Symbiotic associations can have profound

effects on an individual organism, either beneficial or harmful. It is important to study these systems in order to better understand the physiological interactions between species. However, it is often difficult to study individual symbiotic relationships within complex microbial communities. The digestive tract of the medicinal leech, *Hirudo verbana*, presents an ideal system to identify genes required for colonization as it contains a simple, and thus more specific, microbial system. Two species of bacteria dominate the leech crop microbiota, *Aeromonas veronii* and a *Rikenella*-like bacterium. This was discovered by a culture-independent study that looked at the 16S rRNA gene sequences within the leech (Worthen *et al.*, 2006).

The genus *Hirudo* includes the closely related species *H. verbana*, *H. medicinalis*, and *H. orientalis* (Utevsky and Trontelj, 2005). The basic anatomy of the leech consists of a pharynx, crop, intestine, and bladders along with an anterior and posterior sucker. The posterior sucker is used for locomotion, while the anterior sucker, known as the oral sucker, is used to attach to the host and contains chemoreceptors used to identify mammalian hosts (Sawyer, 1986). When a leech consumes a vertebrate blood meal, the blood enters the crop as it feeds by peristaltic contractions of the pharynx (Lent *et al.*, 1988). As the leech feeds, its salivary glands secrete various compounds to aid in the consumption of blood. The main anticoagulant, hirudin, is an anti-protease and an inhibitor of platelet aggregation (Eldor *et al.*, 1996; Rigbi *et al.*, 1987). The salivary secretions also contain hyaluronidase (a spreading factor) and a histamine-like vasodilator (Kraemer *et al.*, 1988). Due to these properties, the FDA has approved the medicinal leech in therapy for post-surgical venous congestion. The leech is able to consume greater than six times its body weight in a single feeding. The lack of proteolytic enzymes in the digestive tract of the leech indicates that the leech symbionts

are responsible for digestion (Mann, 1962). Salts and water are absorbed in the crop where the blood meal is stored. The erythrocytes stay intact for months after feeding (Mann, 1962). In addition to the simple morphology, leeches are relatively inexpensive, and *A. veronii* can be genetically manipulated (Graf *et al.*, 2006).

Aeromonas veronii is a motile, Gram-negative, rod-shaped bacterium. This facultative anaerobe is catalase and oxidase positive, and found in aquatic habitats (Maza *et al.*, 2004). Initial DNA hybridization experiments of the *Aeromonas* genus yielded 12 hybridization groups (HG) of *Aeromonas* species, and further classification proved that the genetically identical HG8 and HG10 are *A. veronii* (Bose, 2006). *A. veronii* is a human pathogen that can cause wound infections, diarrhea, and septicemia. Gastrointestinal illness is particularly common in very young children, elderly, burn patients, and immunocompromised people (Maza *et al.*, 2004). Wound infections following leech therapy can be avoided by administering a prophylactic antibiotic treatment, such as fluoroquinolones (Bauters *et al.*, 2007). However, it is important to fully understand the symbiotic interaction between *A. veronii* and *H. verbana* since leeches are used clinically on patients.

A. veronii is the dominant culturable symbiont in the crop (Graf, 1999). When the leech consumes a blood meal, the blood that enters the crop contains antimicrobial properties, such as an active complement system. The membrane-attack complex (MAC) of the complement system disrupts a cell membrane by forming pores in the membrane, which leads to cell death. Thus, the MAC prevents sensitive bacteria from colonizing the medicinal leech (Indergand and Graf, 2000). To protect *A. veronii* from the complement system, the bacterium has a tetragonally arranged S-layer (surface protein array) surrounding the antigenic lipopolysaccharides of the cell (Kostrzynska *et al.*, 1992;

Indergand and Graf, 2000). The importance of avoiding the complement system was further demonstrated in a study that created serum-sensitive *A. veronii* mutants that were not able to colonize the leech (Brashler *et al.*, 2003). An operating type III secretion system (T3SS) is another characteristic of *A. veronii* that is important for its ability to colonize the leech, as well as a pathogenicity factor (Silver *et al.*, 2007a). The successful colonization of the leech by *A. veronii* results in a beneficial association to the leech. *A. veronii* produces hydrolytic enzymes in the digestive tract, which may assist in the digestion of nutrients (Graf, 2006).

To further discover *A. veronii* genes required for colonization of the leech digestive tract, a signature-tagged mutagenesis (STM) study was performed (Silver *et al.*, 2007b). STM utilizes transposons, which are essentially jumping pieces of DNA to interrupt gene function at a random place in the genome. The advantage to STM is that it allows one to screen for thousands of mutants using relatively few animals. Since each transposon has a unique sequence, the mutants can be tracked by an input and output pool to see which mutants have a decreased ability to colonize the leech. A mutant with a decreased ability to colonize the leech may indicate that the interrupted gene is important for digestive tract colonization. Mutants were verified by testing those with decreased output signals in duplicate (Silver *et al.*, 2007b). One of the serum-resistant mutants, JG573, had a disruption in a hypothetical protein. This mutant had a 100-fold reduced CI value than the wild type and a statistically significant decreased capability to colonize the leech crop after 42 h (Silver *et al.*, 2007b).

This research focuses on characterizing mutant JG573. The disrupted gene is predicted to encode a hypothetical protein. A hypothetical protein is a protein that has no known function but is predicted to exist by computer analysis. In fact, 30% of predicted

genes encode hypothetical proteins, which make them of significant value to study. It is quite difficult to identify the function of a hypothetical protein without a known phenotype. Therefore, knowing the phenotype of a hypothetical protein gives an advantage to identifying its function. For this project, the phenotype of the hypothetical protein is the decreased ability to colonize the leech. Both genotypic and phenotypic experiments can be performed to characterize a hypothetical protein. This may lead to information on the type of protein the gene encodes as well as the physical characteristics of this mutant *in vitro* and *in vivo*.

Sequencing results indicated the disrupted gene in JG573 encodes for an Rhs family protein. *Rhs* stands for recombination hot spot. This name is appropriate for these proteins as they contain repetitive sequences, and these sequence similarities lend to chromosomal reshuffling. The *rhs* elements are large bacterial sequence repetitions that were first found in *Escherichia coli*. *Rhs* elements were discovered when a study showed sequence homology between *rhsA* and *rhsB* was responsible for the *recA*-dependent unequal recombination that leads to the *glyS xyl* duplication of *E. coli* (Capage and Hill, 1979; Lin *et al.*, 1984).

The number of these accessory sequences naturally distributed through *E. coli* varies as some *E. coli* have multiple *rhs* elements (i.e. *E. coli* K-12) while other types of *E. coli* do not have any (Feulner *et al.*, 1990; Hill, 1999). There are eight different *rhs* elements, *RhsA-RhsH*, based on chromosomal location in the *E. coli* genome (Hill *et al.*, 1994; Wang *et al.*, 1998). Each element contains a conserved 3.7 kb core region with an elevated G+C content (about 61-64%) and flanking variable core-extensions with reduced G+C content (<40%) (Feulner *et al.*, 1990; Hill, 1999; Zhao and Hill, 1995). The core has a single open reading frame, beginning at the first base of the core homology and

extending past the core into the variable core extensions (Feulner *et al.*, 1990). In addition to the variation in core extensions (10 different core extensions), the same core extension can be found with a different *rhs* element in separate strains (Hill *et al.*, 1995; Zhao and Hill, 1995). A much shorter, second open reading frame is found slightly downstream of the core extensions, and has 280 codons with a reduced G+C content of 35.8% (Feulner *et al.* 1990). Based on sequence analysis, it is possible that this A+T rich region codes for a signal peptide, which could aid in the export of the core protein to the surface of the cell (Hill *et al.*, 1994; Zhao *et al.*, 1993). Insertion sequences (ISs) have also been found to the right of the downstream open reading frame, including H-rpt (Hinc repeat) found in *rhsB*, *rhsC* (defective copy), *rhsE*, and *rhsF*, and homologs of IS1 (Wang *et al.*, 1998; Zhao *et al.*, 1993; Zhao and Hill, 1995). The core and core extension encode for up to a 160 kDa protein product with a 141 kDa conserved amino end portion and a smaller variable carboxy terminal (Hill *et al.*, 1994). The peptide motif xxGxxxRYxYDxxGRL(I/T)xxxx is repeated 28 times in the core region (Hill *et al.*, 1994; Zhao *et al.*, 1993). Sequence divergence of the *rhs* cores led to three different subfamilies which diverge 22-29%: *RhsA-B-C-F*, *RhsD-E*, and *RhsG-H* (Wang *et al.*, 1998).

The G+C content normally found in *E. coli* is ~50% (Poland, 2009). This is quite different from the conserved core with G+C content ~61-64%, and the core extension with <40% G+C content. Based on the G+C contents and sequence divergence between different *rhs* elements, it is possible that these genetic components were combined, and then obtained by *E. coli* through horizontal gene transfer (Zhao *et al.*, 1993). The same YD repeats found in the *rhs* genes have also been found in teneurin genes, possibly indicating that *rhs* elements descended from these genes (Minet *et al.*, 2000). Based on

phylogenetic analysis, it is possible that a species from the vertebrate or insect taxon carried a teneurin gene with YD-repeats, which then evolved into the three subfamilies of *rhs* elements. *E. coli* then obtained these independent *rhs* core subtypes through horizontal gene transfer (Minet *et al.*, 2000).

Despite everything that is known about the genetic structure of the *rhs* elements, their function is poorly understood. The maintenance of polymorphisms in *rhs* elements may indicate that positive selection is occurring (Peterson *et al.*, 2007). Positive selection most often occurs in cell surface proteins that interact with factors such as the host defense system (Peterson *et al.*, 2007). The function of the large open reading frames in the *rhs* elements is not well understood, although it may produce a cell surface protein. Further evidence indicating that *rhs* elements may encode for a surface binding protein is the repetition of the primary sequence, the hydrophilicity of the core protein, and the large molecular weight of the protein (Hill *et al.*, 1994). *Rhs* elements with sequence homology to the cores are also found in other bacteria species including *Yersinia pestis*, *Salmonella typhi*, and *Pseudomonas aeruginosa* (Hill, 1999).

III. MATERIALS AND METHODS

3.1 Strains

Table 1 lists the bacterial strains used in this research. HM21R is a spontaneous rifampin resistant mutant of the wild-type, HM21, previously isolated from *H. medicinalis* (Graf, 1999). HM21S is a spontaneous streptomycin resistant mutant of HM21R (Rio *et al.*, 2007). The JG573 strains came from the STM study performed on the parent strain, HM21R (Silver *et al.*, 2007). These strains were stored at -80°C in cryovials. *A. veronii* strains were incubated at 30°C, and *E. coli* strains were incubated at 37°C.

Name of Strain	Fosmid	Species	Phenotype	Source
HM21R	-	<i>A. veronii</i>	Rf ^r	Graf
HM21S	-	<i>A. veronii</i>	Sm ^r	Rio <i>et al.</i>
JG573	-	<i>A. veronii</i>	Km ^r , Rf ^r	Silver <i>et al.</i>
EPI300 TM -T1 ^R	pJG573-1	<i>E. coli</i>	Km ^r , Cm ^r	Graf unpublished
EPI300 TM -T1 ^R	pJG573-2	<i>E. coli</i>	Km ^r , Cm ^r	Graf unpublished
EPI300 TM -T1 ^R	pKV-2a11	<i>E. coli</i>	Km ^r , Cm ^r	This study

EPI300 TM -T1 ^R	pKV-2c8	<i>E. coli</i>	Km ^r , Cm ^r	This study
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Table 1. Bacterial Strains

3.2 Media Preparation

LB broth was prepared by adding 10 g BactoTM tryptone, 5 g BactoTM yeast extract, 10 g NaCl, and 1 L deionized water to an Erlenmeyer flask, mixing, and then autoclaving for 15 min at 121°C and 15 psi. LB plates were prepared in the same way as LB broth, except 15 g BactoTM agar was added before the media was autoclaved. The media was cooled to about 52°C after autoclaving, and then antibiotics were aseptically added before plates were poured.

3.3 JG573 DNA Isolation

Two fosmids, pJG573-1 and pJG573-2, were used to sequence the DNA flanking the transposon insertion of JG573. The strains were streaked out from frozen stocks onto LB plates with 50 µg/ml kanamycin and 12.5 µg/ml chloramphenicol, and incubated overnight in a 37°C incubator so that individual colonies were formed. Overnights were prepared by aseptically adding 5 ml LB broth, 50 µg/ml kanamycin (Km), 12.5 µg/ml chloramphenicol (Cm), and a pJG573-1 or pJG573-2 colony to a sterile glass tube. The tubes were then placed in a 37°C incubator shaking at 225 rpm overnight. After 16 h, the overnights were removed from the incubator. To a 14 ml sterile tube, 500 µl of the overnight culture, 1 ml LB broth, and 1.5 µl inducing solution were added. There were 24 tubes prepared to maximize the amount of DNA isolated. The tubes were put back in the 37°C incubator shaking at 225 rpm for 5 h. Five hours later, the culture from the 14 ml tubes were each poured into separate 1.5 ml microcentrifuge tubes. The

Epicentre protocol for the FosmidMAX™ DNA Purification Kit was then followed to isolate the fosmid DNA. To check for DNA isolation, a 0.8% agarose gel was prepared by using 30 ml 0.5x TBE, 0.24 g SeaKem LE agarose, and 2 µl ethidium bromide. The samples were prepared by adding 1 µl JG573 DNA sample, 4 µl nanopure water, and 2 µl bromophenol blue loading dye to a 0.5 ml microcentrifuge tube. Once the samples were loaded onto the gel, the gel was run for 30 min at 121 V. The DNA was stained with ethidium bromide and visualized with an Alpha Innotech FluroChem gel documentation system.

3.4 DNA Sequencing

To identify the gene interrupted by the transposon from signature-tagged mutagenesis, the transposon insertion region was sequenced using a few different techniques. The first approach to sequencing was primer walking. This process involved sequencing several hundred base pairs at a time and using those newly sequenced bases to construct new primers for further sequencing (Table 2). Sequencing reactions were prepared by adding 0.5 µl primer (50 µM), 5.5 µl template, and 4 µl Big Dye v1.1 to PCR tubes. The sequencing program conditions in the thermal cycler were as follows: (i) 65 cycles of 30 s at 95°C, 20 s at 50°C, 4 min at 65°C; (ii) 4°C forever. Once the sequencing program was complete, the samples were precipitated. To do this, first each sample was transferred to a 1.5 ml microcentrifuge tube along with 8 µl nanopure water and 32 µl 95% ethanol. After vortexing, the tubes were left at room temperature for 15 min. Then the tubes were spun in a refrigerated microcentrifuge at 4°C and maximum speed for 20 min. The supernatant was removed, and 125 µl of chilled 70% ethanol was

added to each tube and vortexed. The tubes were spun at 4°C and max speed for 10 min, the supernatant was removed, and the samples were heated at 95°C for 5 min with their lids open. Upon completion of the precipitation protocol, the samples were stored in the fridge until they were sequenced using the Sanger sequencing machine in Beech Hall. Sequence results were analyzed using STADEN.

Sub-cloning was used as a second approach to sequence JG573. Sonication was used to shear the pJG573-1 genome. To a 1.5ml microcentrifuge tube, I added 20µl purified JG573 DNA and 180µl Nanopure water to bring the reaction volume up to 200µl. Then the Gage Lab sonicator was used at level 2 to sonicate the DNA for 5 s. Following sonication, a 0.8% SeaKem Gold Agarose gel was prepared to run out a 1kb ladder and five lanes with 10µl of the sonicated DNA/lane for 60 minutes at 100V. By using a UV lamp and the 1kb ladder as a guide, the 3kb DNA fragments were extracted with a 15-blade. The QIAquick gel extraction kit was used to purify the 3kb DNA, and the final DNA concentration was measured with the Nyholm lab NanoDrop spectrophotometer.

A third approach utilized the EZ-Tn5 <Tet-1> Insertion Kit. The *in vitro* transposon insertion reaction mixture was prepared by adding the materials in the following order: 1 µl EZ-Tn5 10x reaction buffer, 0.2 µg target DNA (0.27 µl of 748.3 ng/µl JG573 DNA), 0.08 µl molar equivalent EZ-Tn5 <Tet-1> Transposon, 7.41 µl sterile water, and 1 µl EZ-Tn5 Transposase for a total reaction volume of 10 µl. The reaction mixture was incubated for 2 h at 37°C. To stop the reaction, 1 µl EZ-Tn5 10x Stop Solution Mix was added to the reaction mixture and then heated for 10 min at 70°C. Transformation and recovery steps were completed according to the Epicentre protocol by electroporation of Transformax EC100 Electrocompetent *E. coli* at 2380 V for 6 ms. Portions of the cells (50 µl, 100 µl, 150 µl, and 200 µl) were plated onto LB plates

containing 10 µg/ml tetracycline. The plates were then incubated overnight at 37°C. DNA was isolated from the colonies by performing a fosmid prep (see JG573 DNA isolation, section 3.3), and then the DNA was sequenced.

The NCBI public database, BLAST (Basic Local Alignment Search Tool), was used to analyze the sequencing results. A BLASTn search was used to confirm that the nucleotides were of the *Aeromonas* genus, and a BLASTx search was performed to see if the sequencing results of the hypothetical protein were similar to any known proteins in the database.

Table 2. Primers

Primer Name	Sequence	Orientation on JG573 genome	Annealed Correctly
P6	5'-ATCCTACAACCTCAAGC-3'	Reverse (3'-5')	Yes
P7	CATGGATCCCATTCTAACCAAGC	Forward (5'-3')	Yes
kv1	AGGGGTCATAGCGGTAAC	Forward	No sequence ^a
kv11	CAAATTGGGTGGTCTGAG	Forward	No sequence
kv12	AAGCCATCCCACTCAAAT	Forward	No sequence
kv13	ATCAGCAAATTCCAACAATC	Reverse	Yes
kv14	TAATGGTACGCCAAGAGAAC	Reverse	Yes
kv16	GTTCCAACACCTCTACAAAAGAG	Forward	Yes
kv17	GTAATCGCATAAACCTTCCCAC	Forward	Yes
kv18	CGAGGCCAGTAGAATCTCTTTC	Forward	Yes
kv19	ATGGCTAATGTTGGTGGTGATG	Reverse	No sequence
kv20	TTGAGTACGCAGGGTCTGGTC	Forward	No ^b
kv21	GATATCAAGGACCTAAGGCGG	Reverse	Yes

kv22	TTTACTCATGGATGTGGAGCATC	Reverse	Yes
kv23	ATGGCATCTGCGCGATAGTTG	Forward	Yes
kv24	GTTACCGCTATGACCCCTTTG	Reverse	Yes
kv25	TGCCGGTGTAAGTGATAACTG	Forward	Yes
kv26	TAACTCAGTGGTGGGGGAAGG	Reverse	Yes
kv27	TGGGTCAATTCGAGTCTGATATG	Forward	<i>No</i>
kv28	ATCGGGCAAGATGACCCGTTG	Forward	Yes
kv29	TTGAGTACGCAGGGTCTGGTC	Forward	<i>No</i>
kv30	CCTGGAACCGGTATTGCGTAG	Forward	<i>No</i>
kv31	CGGGGACATTAACATTGATTCC	Forward	Yes
kv32	GTTTGTGGACGATAGGGTTTTTG	Reverse	Yes
kv33	CTTAGGTATTGGCATGCAAGATG	Reverse	Yes
kv34	CAGGGGCAGATTTATGACCAG	Reverse	No sequence
kv35	ATCGGGCAAGATGACCCGTTG	Forward	Yes

^aNo usable sequence was obtained from the sequencing reaction

^bThe primer did not anneal at the correct location

3. 5 Confirm Location of Transposon Insertion

PCR was used to amplify the expected transposon region. Each reaction was set-up as follows in PCR tubes: 12.5µl Go Taq, 1µl forward primer (kv18), 1µl reverse primer (kv26), 9.5µl nuclease free water and 1µl of ~100ng/µl template (either JG573 or HM21R) for a total of 25µl/reaction. Eight reactions were set-up per strain, and the reactions were placed in a graded thermal cycler. Since the annealing temperature was 63°C for kv26 and 59°C for kv18, a range of annealing temperatures was used to find the

optimum annealing temperature for this primer set. The thermal cycler amplification conditions were as follows: (i) 5 min at 95°C; (ii) 30 cycles of 30 s at 95°C, 30 s at 59°C-68°C, and 3.5 min at 72°C; (iii) 5 min at 72°C; (iv) 4°C forever. Once the PCR reaction was complete, a 0.8% agarose gel was prepared using 30 ml 0.5x TBE and 2.5µl ethidium bromide. A ladder sample was prepared in a 0.5 ml microcentrifuge tube using 0.5 µl 1 kb ladder, 1.5 µl nanopure water and 2.0 µl loading dye. The PCR samples were prepared in a 0.5 ml microcentrifuge tube using 3µl of the designated sample. Loading dye was not added to the PCR samples, as the GoTaq had loading dye pre-added to it. The gel was run for 50 min at 100 V.

PCR products were purified using the QIAquick PCR purification kit according to the manufacturer's instructions. To sequence the PCR products, the following reactions were set-up in PCR tubes: 0.8 µl primer (1 µM), ~50 ng template, 0.75 µl Big Dye v1.1, and X µl nanopure water for a final volume of 5 µl. The sequencing program conditions in the thermal cycler were as follows: (i) 5 min at 95°C; (ii) 24 cycles of 30 s at 95°C, 20 s at 55°C, and 4 min at 60°C; (iii) 10 min at 72°C; (iv) 4°C forever. The samples were precipitated upon completion of the sequencing program in the thermal cycler. For precipitation, each sample was transferred to a separate 1.5 ml microcentrifuge, and then 4 µl nanopure water and 16 µl 95% ethanol were added to each sample. After vortexing, the reactions were left out at room temperature for 15 min. The protocol for precipitating the samples was then completed with the same steps as for the fosmid DNA samples (see DNA sequencing, section 3.4).

3.6 Isolate Wild Type *Rhs* Gene

The wild type gene was obtained from a fosmid library containing HM21 DNA (Silver *et al.*, 2007b). Fosmid library DNA was extracted as previously described in section 3.3. The fosmid library DNA was then screened for the wild type *rhs* gene by using primers kv18 and kv26 (Table 2). Reaction mixtures were set-up in PCR tubes in the following order: 12.5 µl Go Taq, 1 µl primer kv18, 1 µl primer kv26, 9.5 µl nuclease free water, and 1 µl template for a total reaction volume of 25 µl. The program conditions in the thermal cycler were completed as follows: (i) 5 min at 95°C; (ii) 30 cycles of 30 s at 95°C, 30 s at 61°C, 2 min at 72°C; (iii) 5 min at 72°C; (iv) 4 °C forever. A 0.8% agarose gel was prepared using 30 ml 0.5x TBE and 2.5 µl EtBr. A ladder sample was set-up in a 0.5 ml microcentrifuge using 0.5 µl 1 kb ladder, 1.5 µl nanopure water, and 2.0 µl loading dye, and then loaded onto the gel. Then 3 µl of each sample was loaded onto separate lanes, and the gel was run for 50 min at 100 V. The DNA was stained with ethidium bromide and visualized with an Alpha Innotech FluroChem gel documentation system.

The extracted fosmid library DNA was sequenced as described in section 3.4.

3.7 Experiments with Fosmid JG573 DNA and Wild Type *Rhs*

A restriction digest of the mutant and wild type fosmid was performed using the restriction endonucleases Hind III and Sph I. Digests were set-up in 0.5 ml microcentrifuge tube as follows: 5 µg chromosomal DNA, 2 µl Buffer 2, 2.0 µl restriction enzyme(s) (1.5 µl overnight and 0.5 µl next morning), and x µl nanopure water for a total reaction volume of 20 µl. A total of 11 reactions were set-up: 4 with Hind III and the wild type or JG573, 4 with Sph I and the wild type or JG573, and 3 with Hind III + Sph I and the wild type or JG573. The reactions were incubated in a 37°C water bath overnight

for 15.5 h and then spiked with additional enzyme for 3.5 h the following morning, which gave a total incubation time of 19 h. The enzymes in the digest samples were heat inactivated by placing them in an incubator for 20 min at 65°C. A 0.6% agarose test gel of the restriction digests was prepared using SeaKem LE agarose, 30 ml 0.5x TBE, and 2.0 µl ethidium bromide. A 1 kb ladder was used, and 3 µl of each restriction digest + 2 µl loading dye were added to separate lanes. The gel was run at 110 V for 30 min.

A 0.6% agarose gel was prepared for a southern blot in the same way the test gel was prepared except that 11 µl of each digest sample was loaded. The gel was run for 2 h at 50 V. A VacuGene XL blotting unit, Nylon positively charged membrane, SSC, depurination solution (0.25 M HCl), denaturing solution (0.5 M NaOH, 1.5 M NaCl), neutralizing solution (0.5 M Tris-HCl pH 7.5, 1.5 M NaCl), and Whatmann paper were used to transfer the DNA from the gel to Whatmann paper. A picture of the gel, using an Alpha Innotech FluroChem gel documentation system, was taken after the transfer to confirm that the DNA was fully transferred to the Whatmann paper. A labeled probe was prepared using 10 ng/µl of a ~2.5 kb fragment of JG573 containing the *rhs* gene. Hybridization and stringency washes were performed on the blot. Then detection reagents 1 and 2 were used for signal detection, and the blot was exposed for 20 min using an Alpha Innotech FluroChem gel documentation system.

Based on the Southern results, further restriction digests and sub-cloning experiments were performed. Restriction digests using Hind III + pKV-2a11 and Hind III + pBC (pBluescript vector) were set-up using: 3.0 µl chromosomal DNA, 2 µl Buffer 2, 1.5 µl Hind III overnight + 0.5 µl Hind III in morning to spike, and 13 µl nanopure water. Restriction digests with SphI + JG573 and Hind III + Sph I + JG573 were set-up as follows: 3.5 µl chromosomal DNA, 2 µl Buffer 2, 1.50 ul enzyme(s) overnight + 0.5 µl

enzyme(s) in morning, and 12.5 µl nanopure water. All reactions were incubated at 37°C. The pBC digested with Hind III was dephosphorylated by adding the following to a 0.5 ml microcentrifuge tube: 5 µl vector, 2 µl dephosphorylation buffer, and 2 µl SAP (phosphatase alkaline, shrimp). The reaction was incubated for 10 min at 37°C, and then the SAP was inactivated by incubating the reaction for 15 min at 65°C. The ligation reaction for the digested pKV-2a11 and digested (and dephosphorylated) pBC was set-up in a 0.5 ml microcentrifuge tube as follows: 1 µl T4 ligation buffer, 1 µl T4 DNA ligase, 0.5 µl insert (pKV-2a11), 2 µl vector (pBC), and 5.5 µl water. The ligation reactions for Sph I + JG573 and Sph I + Hind III + JG573 were prepared in 0.5 ml microcentrifuge tubes using the TA cloning kit protocol: 0.25 µl fresh PCR product (digestion), 1 µl 10x ligation buffer, 2 µl pCR 2.1 vector (25 ng/µl), 5.75 µl nanopure water, and 1 µl T4 DNA ligase (4.0 Weiss Units). All of these reactions were incubated overnight at 16°C, and then stored at -20°C.

To transform the ligation reactions, TOP10 cells were used, and the One Shot Transformation Protocol from the TA Cloning Kit was completed. Transformations for the Sph I + JG573 insert and Hind III + Sph I + JG573 insert were spread onto LB plates containing 50 µg/ml Km and 40 µl of 40 mg/ml XGal. The transformation for the Hind III digested pKV-2a11 + pBC insert was spread onto LB + Cm 12.5 µg/ml + 40 µl of 40 mg/ml XGal. Three plates were spread for each sub-cloning reaction: 50 µl transformation, 100 µl transformation, and 150 µl transformation. Plates were incubated overnight at 37°C. Isolated colonies were grown up by preparing overnights in sterile tubes, and aseptically adding 5 ml LB broth, antibiotics (Km 50 or Cm 12.5), and a colony. Overnights were incubated shaking at 220 rpm for 16 h at 37°C. To purify the plasmid DNA from the overnights, the QIAprep Spin Miniprep Kit Protocol was

completed. Samples were sequenced and precipitated using the same protocol as for the PCR products (see confirm location of transposon insertion, section 3.5).

3.8 SDS Sensitivity Assay

JG573 and HM21R were streaked out onto LB + Km 100 µg/ml + Rifampin (Rf) 20 µg/ml and LB + Rf 20 µg/ml plates, respectively, from frozen culture. Overnights were prepared in sterile test tubes by aseptically adding 5 ml LB broth, Rf 20 (and Km 100 for JG573), and a colony. The overnights were incubated for 16 h at 30°C. To subculture the overnights, 5 ml LB broth and 50 µl overnighter were added to a sterile test tube. The tubes were incubated at 30°C, shaking at 200 rpm, until the cells reached mid-log phase, OD 600 nm reading between 0.3 – 0.4. Then a 96-well microtiter plate was used to set-up the reactions: 2.5 µl subculture in 122.5 µl LB with or without SDS. The SDS concentrations used were: 0%, 0.3125%, 0.625%, 1.25%, 2.5%, and 5%. For each of the 2 strains, HM21R and JG573, the 6 different SDS concentrations were set-up in quintuplicate. The Microplate Reader in the Gage lab was used to read the absorbance of the plates at 595 nm before and after incubation. The plate was incubated overnight at 30°C shaking at 200 rpm.

3.9 Growth Curve

Overnights of JG573 and HM21R were prepared in the same way as for the SDS sensitivity assay. After 16 h of incubation at 30°C, shaking at 200 rpm, the overnights were sub-cultured to mid-log phase using the same procedure as the SDS sensitivity assay. A 48-well plate was used to set-up 6 reactions in duplicate: LB (blank), LB + 0.3125% SDS (blank), LB + HM21R, LB + 0.3125% SDS + HM21R, LB + JG573,

LB + 0.3125% SDS + JG573. The sub-cultured JG573 and HM21R samples were diluted to 0.100 O/D using LB. Each well was inoculated with 200 µl LB with or without SDS, and 10 µl of the HM21R or JG573 dilutions were added to the appropriate wells. The plate was incubated at 30°C for 24 h, and automatic readings were taken every ten minutes.

3.10 Test for Sensitivity to Antimicrobial Compounds

Biolog plates were used to screen HM21R and JG573 for sensitivity to two hundred-forty different reagents. These plates were pre-coated with a variety of compounds in 96-well plates at four different concentrations per reagent. To inoculate these plates, first frozen cultures of HM21R and JG573 were streaked out onto LB + Rf 20 and LB + Km 100 + Rf 20, respectively. Colonies were resuspended to create a 0.032 Abs cell suspension in LB for each strain. 1.5 ml Biolog Redox Dye Mix A (100x) and 23.5 ml nanopure water were added to the sterile container containing 125 ml IF-10a GN Base Inoculating fluid (1.2x). The container was inverted to mix, and 120 ml were transferred to a sterile vial. One sterile vial was prepared per strain. Then 600 µl cell suspension was added to the vial and carefully mixed by inverting the vial. The mixture was transferred to a sterile petri dish. Each well, for each 96-well plate, was inoculated with 100 µl of the appropriate cell mixture. PM plates 11-20 were inoculated for each strain. The plates were incubated for 24 h at 30°C. If the cell was able to reduce the redox dye as it respired, the well color changed to purple. A clear well indicated that no reaction had occurred.

3.11 Competition Assay

The strains HM21S and JG573 were used in this assay. Overnighters were prepared for each strain by aseptically adding 5 ml LB, Km 100 µg/ml and Rf 20 µg/ml for JG573, Sm 100 µg/ml for HM21S, and a colony. They were incubated at 30°C, shaking at 200 rpm, for 16 h. The overnighters were sub-cultured to mid-log phase using the same procedure as the SDS sensitivity assay and growth curve. 5 ml sheep blood was aliquoted to 15 ml falcon tubes; 1 tube of blood was prepared per leech. An additional 2 ml tube of blood was prepared to use as bait blood. The tubes of blood were placed in a 56°C water bath for 1 h to heat-inactivate the complement system. After 1 h, the water bath was turned down to 37°C to cool the blood. To prepare the leeches, jars were filled half-full with leech water and labeled. Before adding a leech to a jar, the leech was patted dry with a paper towel and weighed. The conversion factor of 3.1×10^8 CFU/ml/OD was used to calculate how much of the overnighter contained 10^6 CFU. This amount was added to a 1.5 ml microcentrifuge tube containing 1 ml 0.8% NaCl so that the cell density was 10^6 CFU/ml. The sample was further diluted by transferring 100 µl from the microcentrifuge tube containing 10^6 CFU/ml to a 1.5 ml microcentrifuge tube with 900 µl 0.8% NaCl. Then the heat-inactivated blood was inoculated with 12.5 µl per strain, so that 250 CFU/ml of the competitor and 250 CFU/ml of the mutant were added to the blood. After inverting the tube several times to mix, 500 µl were transferred to a 1.5 ml microcentrifuge tube on ice until the input blood was plated. The input blood was plated in duplicate on LB + Sm 100 and LB + Km 100 + Rf 20 plates in 100 µl aliquots for each leech. Input blood plates were incubated at 30°C overnight, and the colonies were counted the following day. An additional 500 µl was removed from the inoculated blood, and transferred to a 1.5 ml microcentrifuge to be left out at room temperature during the leech incubation time to serve as a blood control. Once the blood was

inoculated, a piece of parafilm was stretched over the open falcon tube. A sterile needle tip was used to pierce the parafilm. The tube was dipped in the bait blood to serve as bait for the leech. Then the leech was fed the blood, and put back in the jar to incubate for the appropriate amount of time (18, 24 or 42 h). The leeches were weighed about 30 min post-feeding.

After the incubation period, the leeches were weighed. A string was tied around the mouth of the leech to prevent the leech from vomiting the blood meal, and the leech was sedated by placing it in a tube of 70% EtOH. The leech was placed in a sterile petri dish, and cut in the middle using a 15-blade. The intraluminal fluid was pipetted into a 1.5 ml microcentrifuge tube and placed on ice, along with the blood control, until plated. The intraluminal fluid and blood control were considered dilution A. Dilutions B-E were prepared by transferring 50 µl from the previous dilution into a 1.5 ml microcentrifuge tube containing 1 ml 0.8% NaCl and vortexed. Dilution A was plated in 50 µl amounts, and dilutions B-E were plated in 100 µl quantities. Each dilution was plated in duplicate on LB + Sm 100 and LB + Km 100 + Rf 20 plates for each leech. Plates were incubated overnight at 30°C, and then colonies were counted and recorded the following day along with the dilution designation. The colonies were multiplied by the appropriate dilution factor, and colonies from duplicate plates were averaged. The CI value was calculated using the equation: $(M_o/C_o) / (M_I/C_I)$, where M is the mutant JG573, C is the competitor HM21S, O is the output (intraluminal fluid or blood control), and I is the input.

IV. RESULTS

4.1 Sequencing JG573

The initial goal was to sequence more of the DNA flanking the transposon insertion to identify the inactivated gene. First, primers annealing to the transposon, P6 and P7 (Brown, *et al.*, 2000), were used to sequence outwards from where the transposon inserted into the genome. Based on initial sequencing results, subsequent primers were designed by using primer design programs on the Staden network as well as the Primo Sequencing 3.4 (<http://www.changbioscience.com/primo/primoseq.html>) and Web Primer (<http://www.yeastgenome.org/cgi-bin/web-primer>) programs available on the internet. Primers were devised to sequence further out from the transposon like primers P6 and P7 as well as into the expected transposon insertion region to confirm the presence of the transposon. While this is a relatively straightforward process, problems occurred when primers designed for a specific location were annealing to different locations; this is especially challenging when the DNA contains repetitive regions (Table 2). The primers that showed the greatest variation in annealing, including primers that were expected to sequence in the opposite direction, were primers kv20, kv27, kv29 and kv30. In fact, all of these primers annealed so that their sequences were in the expected transposon insertion region. These sequencing results masked the transposon sequence and suggested the presence of repeated DNA sequences.

To further analyze the sequencing results (Figure 1), a BLAST (Basic Local Alignment Search Tool) search was performed on the NCBI database. BLASTn results showed a very high similarity to *Aeromonas salmonicida* with an Expectation value of 0.0 and 93% identities (Figure 2). The results of the BLASTx search yielded a high similarity to *rhs* family protein with an Expectation value of 3×10^{-175} and 91% identities (Figure 2).

GCCCCGTCGCCCCGTGATGCCGTGGTGGAAACCAGTTGTTTCATCAGCAGCATCACCCAGAAGGTAGAGAGCC ATGAGAAATGAGAGACATTGGTGATGGGGTGAGGCGACGTGCCCATATGCAGACTAAGAGGATAGTGG

TGATCAAATCGCCGAGTCCCTCAGTTAATTCCATTCAAGCTGCGTCGATTGTTGGGCAACAACGCCACTTATA
 CTAAAAAATTACAACCTAGAAAACCTATTTTAAGCTATAACAATTCTTTTATCTCAGTTGCATCACCACCA
 ACATTAGCCATTATTTCTTTGTAAAAATGAGGTGAAGTTATCACATAGCCTATATACATCACCTTCTTCATT
 TGTTCCTTTGCGTTCAGCATGCTCTCCAGAGTGCAAGTGCGTTCTATCCCAATAATAAACTGAATCATCAA
 CTCCAGAGCATTCAATAAAAAACATATTGCCACCAGGGTCTGCACCGATAATAAACGGCCTGTTAAACCC
 GACTATTGCCCCCTGAACTGATTGGCATAATTCAGATCGAGTAGCGGATTTACAGTTTTTATTCATACA
 TTTCTGAAAATCAATAACTCCATCATCAACCTTCATAAAAGGAATAGTGCAGTATGAATTGGTGCTAAT
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 GGATCTTTTCTTAATATTAGATGCTCCACATCCATGAGTAAATATTAGCAGGTTCCGCATGAATATCTTTG
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 CTCAGTAACTGTAAAAAACACCGGCAATATGCCGGTGTAAGGTGATAACTAGATCTGTACAGCTAGTCGA
 CGATGATTTCGTAATCAAAAAACCTATCGTCCACAAACATGTAGTGACTATGTGGCATTGTAATAGTCAT
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 GCTTTACAATATCCATGATTATTTTTTTTCTTCTCTGTCTTTTATTATAAAACTTCGAGGCCAGTAGAA
 TCTCTTTCTAGGTTTTCTTCCCGCGGGGACATTAACATTGATTCCACACCATCGACAGATGATTTAGTA
 ACAACTTTTTTTAATGCATTTACAACTCTTCAGATCTCATTGTGGCCTCACTTAATCGACTGTGTTTTCTT
 AAAAGCTTCTGGGAACATCTCCTTGTTAGAGAAATCAGCTCCCTTATCGCTGACGCTGGTGCAATTGGTGT
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 TTGTCCGGCAGGATCCTTTGTACCGCGTGATGTATATCCAATCATCGCCACTTACGGAACGATTCTTTA
 AGTCATTGAACGCTCCAACCTTCATATTTTTTCACTGGGCATCCCGCAACCCAGCGGATCTACCCATTCC
 AGCGGGTTATGCTCATATCCCTGTGGCCTAAGCCCTCCCGCAAAACCTATCGGGTCTGGCGAGATATATT
 GCCCCAACTCCGGGTCAAAGTAACGGTGCCGGTTGTAGTAAAGCCCCGTCTCCTGGTCATAAATCTGCCC
 CTGATAGCGCAGCTCACATAAGTTTCGTCAATTGGCTGCATCCCCAAGGAAGCGCCTGAGCGGTTGTGGG
 ATGGCATCTGCGCGATAGTTGCCCCACAGCCGCTGCTCACCAGCGCCAGATAATGTCACCGGCTTCGCTGC
 ACAGCTCGCGGGTGTTGCCCGCCACGTCCGTCACGATATAGTGACAGACGCTCGTCTGCTCACCAGCTTC
 CCACTGGGCCAGCGGCCTGAAGCTGCCGGGCTCATAGACCCACTGCACCTGACCGGGCGGCTTGCCATCG
 GCCCCGTAGCTTTGTTGCTGCACCAGCTGGTCAACGTCACCGATAGTGAGCGGGTTATCGCGGTGA
 GTTGGCTCACCTGCCCCCTCCCGCCCCCTTGCCGATACGCCGACCAAGGGGTTCATAGCGGTAACGCCAGCG
 GCTGCCATCGGGCAAGATGACCCGTTGCAGTCGGTCAAAGCCATCCCACTCAAATTGGGTGGTCTGAGGC
 CGGAAACCGGGGCGTGATACCACCTTCTCACTGAGCCGTCCGCACTTGTCGTAGCGGTACTGATTTTGGC
 CTATCTGGCTGACCCGGCCCCGACGCTCATAACGGGCGCAAGGGCTCGGCCTGCGCTGCTGGCTGGACAG
 CGGCTGCCCCATCGCTTGGCAGAATATGGGAGAGCTCGCAGAGGTTCACTCGCTGTCATAAGCAACAGC
 CGGGCCTGACGCTCGTGCCATTGAGCTTCCAACCCTGTTACACCCAGTGGCTGTAAAAAACACCGGCA
 ATATGCCGGTGTAAGTGATAACTGGAACAATATAAACTAGGGAAGGTGCGAACAAGTCACTTAACAAG
 AAGTCATATCATCATAACCTATTGATATTGAGTGGCTAGATTCTCAGTCCGTTGTTAACTGAGTACTTATT
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 CCCCAGATAATCACCATCATCACCGAAGATAAAGCTACCAAGAGAAAAATTACCTAAATCATACTTCAA
 AATAAAACGCTTAAATTTATCATGCAACTTAATGTTTAGCTTTTGTTCTAAATCAATAACATTATCAACAG
 ACCCCCTCCGTTTTTTCATGATCAAGTCATCCATTTCCCCACCAAAACGGAGAAAAATTTTTCATATAGCTTA
 GATTCTATTTCTGATAATTCAGCAGCATAAAAAACCTCTCTATTTTCGTATGCCAAATACCACCACTTCCTA
 CATGCGTTTTTCCCTGGAACCGGTATTGCGTAGCGCCCTTGATCAACATGATGATGAATTAATGTATCACCT
 TTGTAATTTGCATGTTTCAGGAAACATTTTAACCCAAGTATCATCTATTTTGGAGACAATCCTAACTCATT
 AACTCTATATGCGTTACTTTTACTCAATGAATCAGGTTGCAAACTACTCCACTGCTTCCAGAATTGTTATT
 ATCTCGAAGACCTCCGTTGCAGTCTTAACCCCTGAGCTAAATGTTGCTGATCAATTGAGTACGCAGGGTC
 TGGTCGCCCTGGAACCTTTTGGGTCAATTCGAGTCTGATATGGATTATACCTCTCGGGATTGACTCTTTAGC
 TTGTGTCTTTGCCCGGACCCACTCAACCCCTGCGG

Figure 1. DNA sequence of JG573.

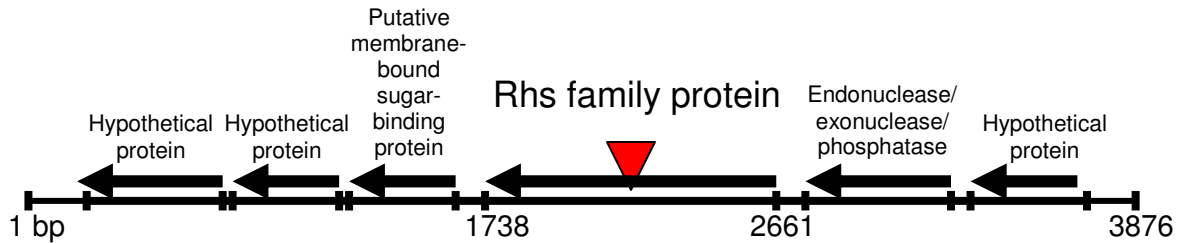


Figure 2. Sequencing results of JG573. Comparing the sequence to the NCBI database of known sequences using BLASTx revealed that the interrupted gene codes for Rhs family protein (Tn indicated by inverted triangle).

Since the repeated DNA sequences hindered the use of sequencing by primer walking, sub-cloning the mutant into a few different types of plasmids was used to try to increase the rate of sequencing. The sonication method took several trials to increase the concentration of DNA fragments extracted. Despite a few attempts of using the sonicated DNA to sub-clone with the TA cloning kit and the Hanahan cell protocol, sub-cloning was unsuccessful. The underlying problem with this method is that the starting JG573 DNA concentration was 1.7 µg/µl, and the final concentration of the purified 3kb fragments was 4.8 ng/µl. This concentration of DNA was not high enough to sub-clone into a vector so this method was stopped.

Since sub-cloning with the DNA extraction was unsuccessful, a different sub-cloning method was used. The EZ-Tn5 <Tet-1> Insertion Kit was chosen because the whole fosmid could be used for this kit, which would theoretically eliminate the problem of trying to concentrate the reduced concentration of DNA from sonication. The colonies that were sequenced from this kit were unsuccessful. In order to understand why the sequence reads did not work, a 0.8% agarose gel was prepared so that the sample could be run out on the gel. Results indicated that the samples contained very little DNA, as indicated by the lack of strong fluorescent signals in the sample lanes (Figure 3).

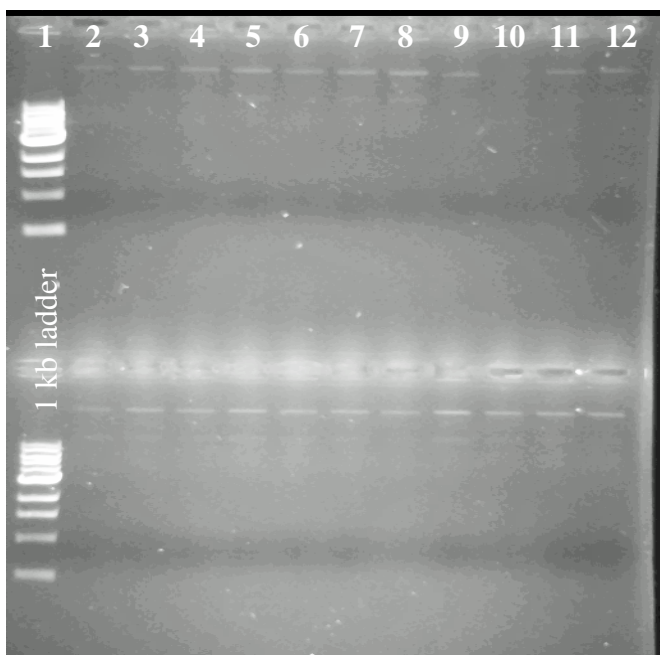


Figure 3. DNA isolated from EZ-Tn5 <Tet-1> Insertion kit. Lane 1 was loaded with the 1kb ladder, and lanes 2-12 were loaded with DNA samples isolated from the colonies.

4.2 Confirm Location of Transposon Insertion

Following identification of the primary sequence of JG573, the next step was to confirm the location of the transposon insertion. PCR was used to verify this since initial sequencing results masked the transposon sequence. The transposon used in STM was a 1.8 kb pUTminiTn5Km2STM plasmid with a unique signature tag (Silver *et al.*, 2007b). Since the parent strain, HM21R, was not an STM mutant, the transposon insertion in JG573 was confirmed by looking for a size difference between HM21R and JG573 through PCR amplifying the expected transposon insertion region. The expected transposon insertion region in JG573 was based on initial sequencing data from primers P6 and P7. Primers kv26 (forward primer) and kv18 (reverse primer) were chosen for PCR since they were designed about 1.8 kb apart, and their sequencing region encompassed the expected transposon region.

PCR products were run out on a gel to analyze the results (Figure 4). Gel results of the PCR products showed a clear size difference between HM21R and JG573. The PCR product from HM21R was about 1.8 kb large. This was expected since the region between primers kv18 and kv26 is approximately 1.8 kb. The predicted size of the PCR product from JG573 was 3.6 kb, as the region between the primers is ~1.8 kb and the transposon size is also 1.8 kb; however the actual size of the band was about 2.5 kb. This is a 0.7 kb size difference between HM21R and JG573. By having only a 0.7 kb size difference between the two strains, it was not possible to determine whether this was due to a partial transposon insertion or some other rearrangement. To ensure that the 0.7 kb was due to the insertion of the transposon, sequencing of the PCR products was performed. A BLASTn search of the sequencing results indicated a very high similarity to transposon delivery vector pUTkm1 (E value = 4×10^{-105}).

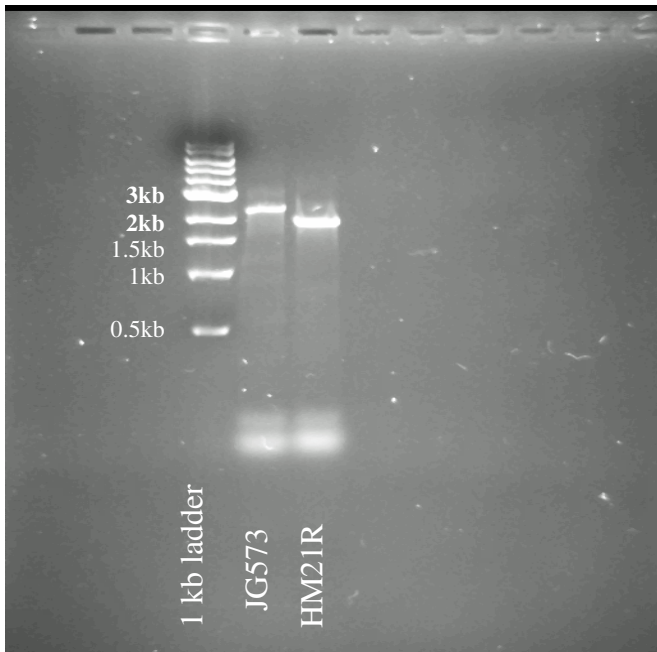


Figure 4. Results for JG573 and HM21R PCR amplified with primers kv26 and kv18.

4.3 Isolate Wild Type *Rhs* Gene

The wild type *rhs* gene was recovered from an ordered fosmid library DNA. This was done by a PCR screen of six microtiter plates to identify which plate(s) contained the gene. A negative control was also prepared to ensure that results were accurate. Plates 2, 4, and 6 showed positive results for containing the *rhs* gene. PCR was then performed on plates 2 and 4, rows a-h. Gel results of these PCR products indicated that rows 2a, 2c, 4a, and 4b all contained the *rhs* gene. A third PCR reaction was performed on rows 2a and 2c, wells 1-12 to isolate which particular wells contained the gene. Gel results showed that wells 2a11 and 2c8 had the *rhs* gene, which were named pKV-2a11 and pKV-2c8, respectively. The extracted Fosmid Library DNA from pKV-2a11 and pKV-2c8 was also used in sequencing with primers kv18, kv24, kv26 and kv31 to confirm that the isolated wells contained the same *rhs* gene as the mutant. Sequencing results showed the wild type sequences lining up with the JG573 contig, which indicated that the isolated gene was the same *rhs* gene as the mutant. Restriction digests also confirmed that the *rhs* gene of the isolated wild type and mutant were the same (Figure 5). Signal detection of the Southern blot showed which bands of the digest contained the *rhs* gene so that further sub-cloning experiments could be designed to sequence more of the *rhs* gene (Figure 6).

Even though isolation of the wild type *rhs* was successful, sequencing the clones from the various transformations with pJG573-1, -2 and pKV-2a11, -2c8 was unsuccessful, and no new sequencing data was obtained. The lack of DNA in sequencing from the sub-cloning experiments appeared to be one of the problems with the sub-cloning experiments for JG573 and the wild type *rhs*. These experiments were also completed in an effort to increase the rate of sequencing, for both the JG573 *rhs* locus and the wild type *rhs* locus. A NanoDrop spectrophotometer was used to measure the

concentration of the DNA isolated from the clones. Results showed a range in DNA concentration from 9.2 ng/μl to 18.9 ng/μl, which may have contributed to the lack of sequencing results. Also, in the ligation reaction between the Hind III digested pKV-2a11 and pBC, the incorrect pBluescript vector was used. PBC has chloramphenicol resistance, and the sub-cloned colonies were grown on LB plates with 12.5 μg/ml Cm. Since the vector was already resistant to the antibiotics on the plate, the lack of DNA for sequencing from clones on those plates may have been due to isolating an empty vector. This experiment could be repeated in the future using pBS, which is pBluescript with ampicillin resistance, to ensure that no empty vectors were able to grow on the LB + Cm12.5 plates.

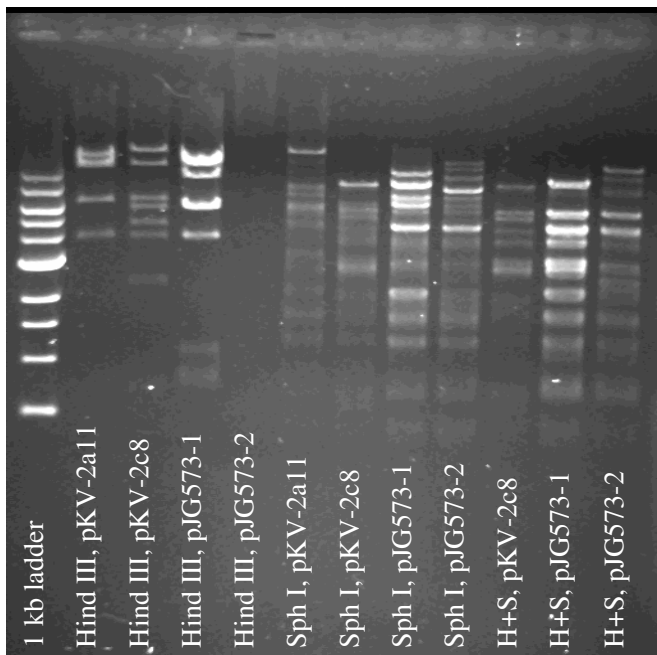


Figure 5. Restriction digest of mutant and wild type *rhs*.

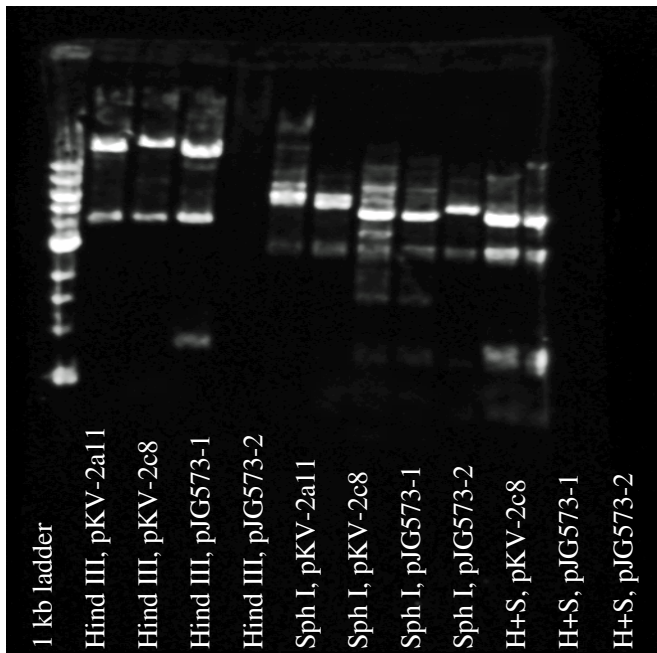


Figure 6. Southern blot after signal detection.

4.4 SDS Sensitivity Assay

Others predicted that *rhs* elements encode cell surface proteins. This raises the possibility that membrane disturbing agents could affect the growth of *rhs* mutants. Sodium dodecyl sulfate was chosen because it is an anionic detergent that acts on the cell surface. Another STM mutant, an *lpp* mutant, was previously shown to be significantly more sensitive to SDS than HM21R (Silver *et al.*, 2007b). To begin identifying *in vitro* phenotypes of the mutant, an SDS sensitivity assay was performed. The SDS sensitivity assay was performed twice to obtain accurate results. Results from the first assay indicated that the differences for the HM21R and JG573 values were statistically significant for 0.3125% SDS, 0.625% SDS, 1.25% SDS, and 2.5% SDS. At 0% SDS, the cell growth was uninhibited, and growth of both strains at 5% SDS was almost completely inhibited. These initial results pointed toward a possible phenotype. When

the experiment was repeated, different results were obtained. Results indicated that there was no significant difference between the parent and the mutant at any of the SDS concentrations (Figure 7). The only difference between the first and second SDS sensitivity assays was that growth occurred two hours longer for the first assay than the second assay. To explore whether this difference was due to a defect in the growth rate of the mutant as compared to the parent, a growth curve experiment was performed.

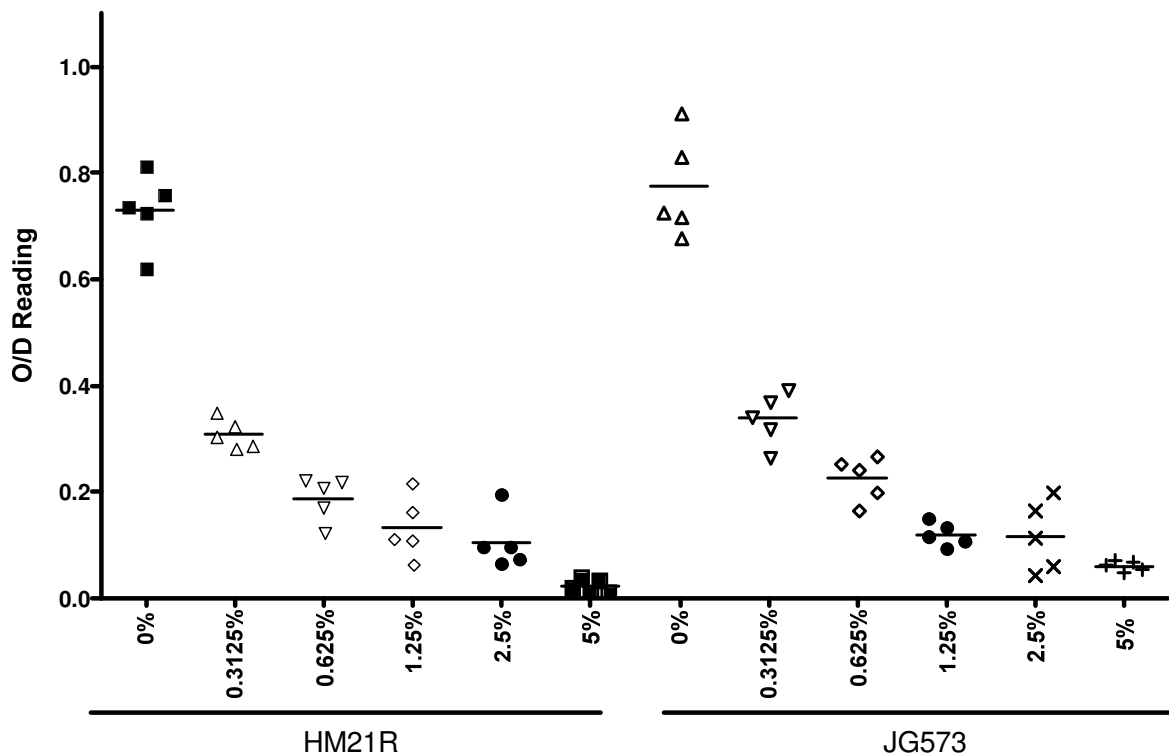


Figure 7. SDS sensitivity assay results. Statistical analysis revealed that there was no significant difference between growth of HM21R and JG573 at any of the concentrations tested.

4.5 Growth Curve

A growth curve experiment was performed in order to compare the growth of JG573 and HM21R in LB and LB + 0.3125% SDS. This was completed to see if the difference in the SDS assay results were due to a defect in the growth rate of JG573 or simply due to a false sensitivity detected in the first assay. The growth curve experiment was completed twice in order to ensure repeatability. Results from both trials indicated that the parent and the mutant follow essentially the same growth curve in both LB and LB + 0.3125% SDS (Figures 8, 9). JG573 did not show any defect in growth, as compared to the parent, over any time point during the 24 h. This means that the incubation time difference for the two SDS assays should not have made any variation in the results. Therefore, the SDS sensitivity assay that showed a defect in the mutant as compared to the parent was a false positive. This is further supported by the fact that JG573 was able to grow to equal levels as HM21R when grown in LB + 0.3125% SDS.

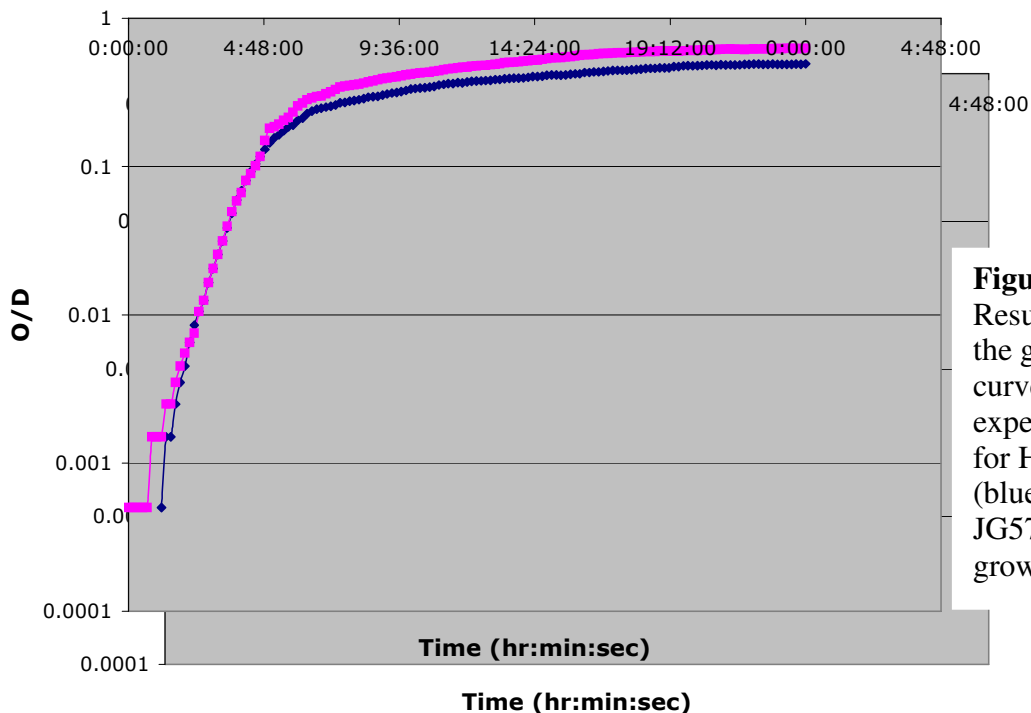


Figure 8. Results of the growth curve experiment for HM21R (blue) and JG573 (pink) grown in LB.

Figure 9. Growth curve results for HM21R (blue) and JG573 (pink) grown in LB + 0.3125% SDS.

4.6 Test for Sensitivity to Antimicrobial Compounds

Biolog plates were used to screen JG573 and HM21R for differences in chemical sensitivity to two hundred-forty reagents. By quickly screening for a variety of antimicrobial compounds, the results could be used to provide more information on the *in vitro* phenotype of JG573. Each compound was pre-coated into four separate wells at increasing concentrations. Therefore, an antimicrobial compound that differed in sensitivity for the parent and the mutant by more than one well could indicate that one of the strains is particularly susceptible to that reagent. Once the Biolog plates were incubated, a positive reaction was indicated by a purple color change since the cell reduced the redox dye as it respired, and a clear well indicated no reaction had occurred (Figures 10, 11). Chemicals with results for JG573 and HM21R that differed by more than one well were recorded (Table 3). As is evident, the parent was sensitive to many more compounds than the mutant. Taking the one well differences between HM21R and JG573 into consideration further demonstrates the increased sensitivity of the parent.

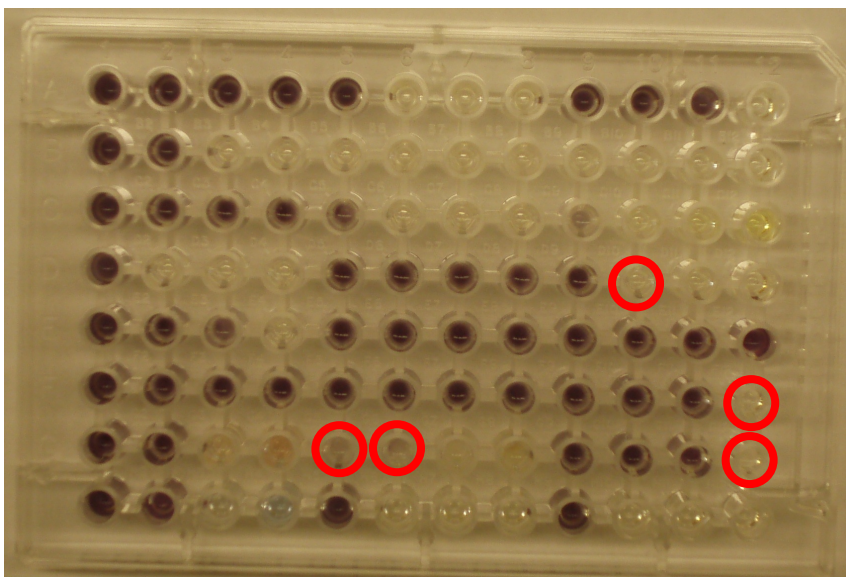


Figure 10. PM Plate 13B inoculated with HM21R. The circled wells indicate wells that were positive for growth in JG573 but absent for growth in HM21R. This difference in growth indicates that HM21R is more sensitive to the chemicals in those wells than JG573.

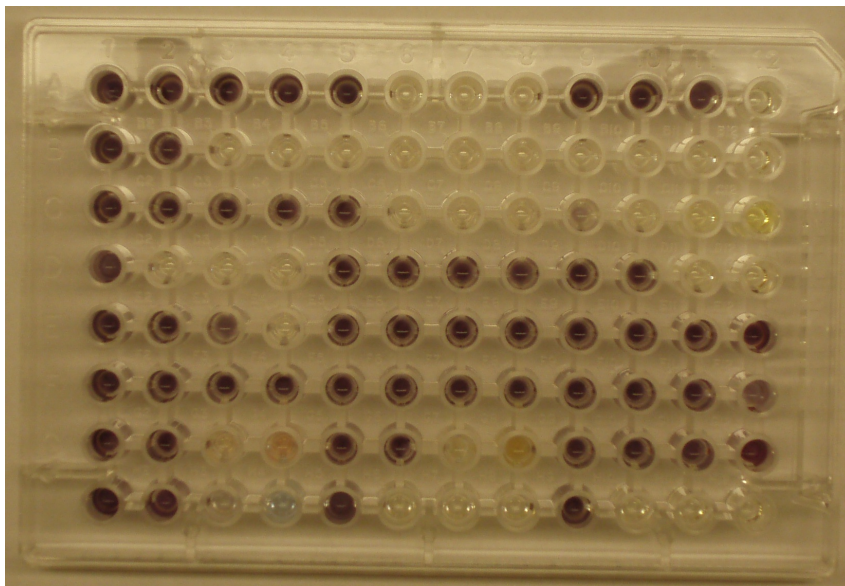


Figure 11. PM Plate 13 B inoculated with JG573.

Plate	Reagent	Strain with increased sensitivity
PM13B	Manganese chloride	HM21R
PM16A	Streptomycin	HM21R
PM16A	Potassium tellurite	JG573
PM18C	Sodium metasilicate	HM21R
PM18C	Myricetin	HM21R
PM18C	2-Phenylphenol	HM21R
PM18C	Plumbagin	HM21R
PM19	Phenyl-methyl-sulfonyl-fluoride	HM21R
PM20B	8-Hydroxy-quinoline	HM21R

Table 3. Biolog PM Plate Results.

4.7 Competition Assay

To further characterize the *in vivo* phenotype of the mutant, a competition assay was performed. This assay was used to see when JG573 begins to have a colonization defect in the leech. The colony counts for HM21S and JG573 from the LB + Sm100 and LB + Km100 + Rf20 plates were used to calculate the CI (competitive index) value. A CI value of less than 1 indicates that there is a defect in the mutant. Three leeches were tested for each time point. The CI value calculated using the input blood and intraluminal fluid was expected to show a defect at some time point, since that would indicate that the mutant has a disruption in a gene that decreases its ability to colonize the leech (as compared to HM21S). CI values were also calculated using the input blood and control blood. The purpose of finding this value was to see if the mutant had a decreased ability to grow in blood, which would indicate a false positive colonization defect in the leech. This was not expected since the interrupted gene in the mutant should have caused a decreased ability to colonize the leech, not increased serum sensitivity. The 24 h competition assay was repeated to ensure accurate results. Results from all of the time points are in Figures 12 and 13. As can be seen in the graphs, results did indicate that the mutant had a significant defect in the leech starting at 24 h which continued at 42 h. However, JG573 was able to grow to almost equal levels of HM21S in the blood, indicating that the mutant did not have a growth defect in blood.

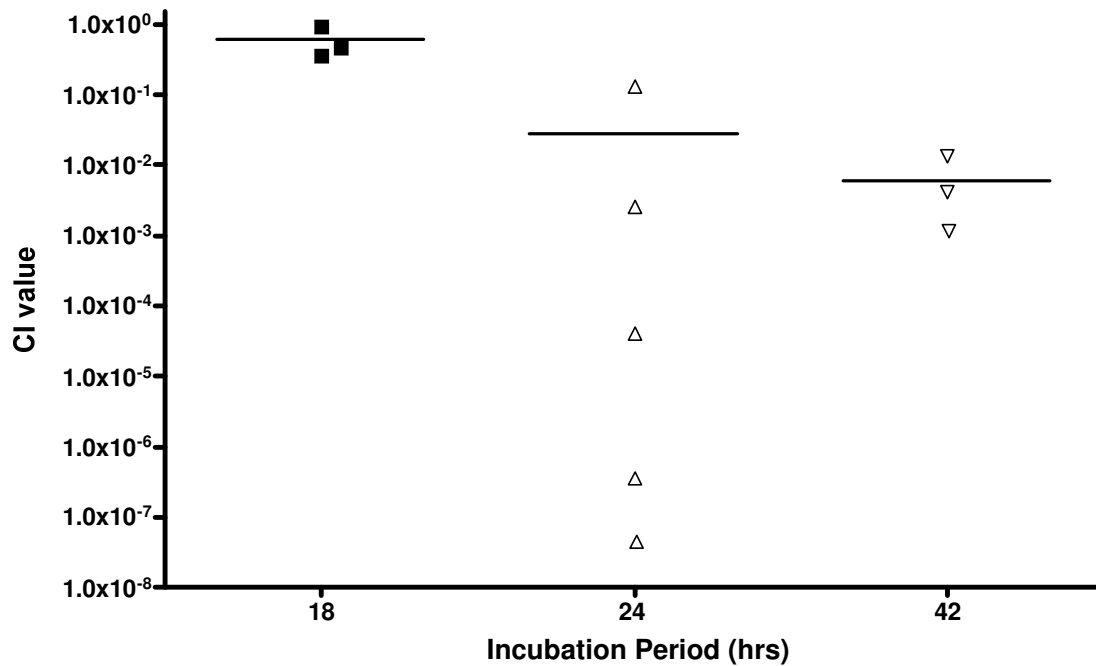


Figure 12. *In vivo* competition assay results. Based on column statistics comparing the CI value to 1.00, JG573 colonized the mutant significantly less than HM21R at 24 and 42 h.

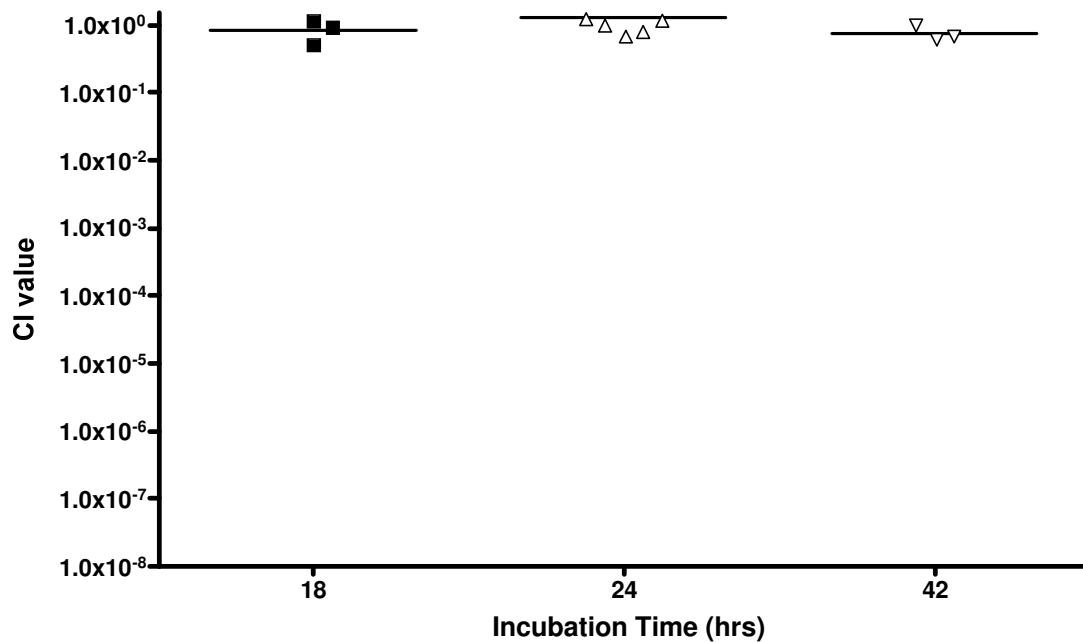


Figure 13. *In vitro* competition assay results. Column statistics demonstrated that there was no significant difference between the CI values for HM21S and JG573 at any of the time points. This indicates that the decreased ability of JG573 to colonize the leech at 24 and 42 h is not due to a growth defect in blood.

V. DISCUSSION

The purpose of characterizing the hypothetical protein, JG573, from signature-tagged mutagenesis was to better understand the symbiotic interaction between *A. veronii* and *H. verbana*. Sequencing results indicated that the gene inactivated in JG573 was most similar to an *rhs* element. Although not much is known about the function of the Rhs protein, the sequencing results provided important information about the interrupted gene in JG573. As stated in the introduction, the *rhs* elements are composed of large sequence repetitions. With multiple large repeats present in the sequence, the STADEN network could have aligned them on top of one another. These proposed repeats could explain why primers kv20, kv27, kv29, and kv30 were unable to anneal in the correct location, and why the transposon sequence was masked. As discussed earlier, the repeated peptide motif YDxxGRL(I/T) is found in the conserved core region of *rhs* elements in *E. coli*. Similar motifs were found at five locations in the *A. veronii rhs*

homolog. Despite the fact that these small repeats are not large enough to mask the transposon sequence, this does provide conclusive evidence that there are repeats in JG573, very similar to the same ones found in the *E. coli* genome. Further inspection of the JG573 genome in the future may reveal even larger sequence repetitions.

Even though the genetic structure of the *rhs* elements has been thoroughly studied, very little is known about the function of the protein. The interruption in the *rhs* gene in JG573 is a novel opportunity to learn more about *rhs* elements because other researchers did not have phenotypes. It is possible that understanding the phenotypic differences between a wild type *rhs* gene, such as that in HM21R, and a mutant in the *rhs* gene, as in JG573, may lead to a better understanding of the functional role that this protein plays both *in vitro* and *in vivo*. A variety of experiments were performed to analyze the phenotype of the mutant. While the SDS assay indicated that JG573 did not have any statistically significant sensitivity to SDS as compared to HM21R, the Biolog plates did reveal some antimicrobial compounds that had sensitivity differences between the parent and the mutant (Table 3). Results indicated that the mutant seems to have acquired a greater resistance to reagents than the parent. Perhaps the *rhs* protein is a membrane pore that may normally allow chemicals to pass through into the cell. The mutation in JG573 may have disrupted the function of the pore, preventing chemicals from getting in the cell as readily. Another possible explanation is that the wild type *rhs* protein is so large that it lets in small antibiotics. Further characterization of this protein through dose response curves to find the minimum inhibitory concentration (MIC) for the various chemicals that differed in sensitivity between HM21R and JG573 may lead to further understanding of the function of the *rhs* protein and its role in the symbiotic relationship between *A. veronii* and *H. verana*.

Biolog Plate test results showed that the only antimicrobial compound that the mutant was more sensitive to than the parent at greater than one concentration was potassium tellurite. The strong oxidant potassium tellurite is particularly toxic to Gram-negative bacteria (Taylor, 1999). Although the mechanism of toxicity is unknown, it may enter the cell through phosphate carriers or iron and monocarboxylate transporters (Castro *et al.*, 2009). The environmental isolate *Aeromonas caviae* ST exhibits tellurite resistance through the *aceE*, *aceF*, and *lpdA* genes that form the pyruvate dehydrogenase (PDH) complex (Castro, *et al.*, 2009). The Biolog plate results will have to be further verified by testing the sensitivity of JG573 and HM21R at various known concentrations of potassium tellurite. Based on these current *in vitro* results, the function of the *rhs* protein could be a membrane surface protein that helps to confer potassium tellurite resistance at the surface.

To test the *in vivo* phenotype of JG573, a competition assay was completed in the leech. The results indicated that the viability of JG573 significantly decreased between 18 h and 24 h in the leech, and this decreased colonization ability was not due to an effect of the serum or blood (Figures 12, 13). This dramatic decrease in colonization was expected since JG573 came from the STM study that screened for mutants with decreased colonization ability inside the leech (Silver *et al.*, 2007b). The decreased colonization ability of JG573 is likely to be attributable to the mutation in the *rhs* gene, but has to be confirmed by complementing with the wild type gene. The 24 h time point *in vivo* showed a large variation in CI values, unlike the 18 h and 42 h time points. This may be because 24 h is a dynamic point when physiological changes occur at slightly different times or to varying degrees, which lead to the observed variation. The onset of this change appears to occur at different time points in different leeches.

Current literature asserts that the *rhs* elements may code for a ligand-binding protein (Hill, *et al.*, 1994). Fluorescence in situ hybridization (FISH) imaging of the leech crop after feeding has revealed that *A. veronii* appears to associate with the microcolonies formed by the *Rikenella*-like bacteria in the intraluminal fluid (Kikuchi and Graf, 2007). It is possible that the *rhs* protein plays a role in binding to the *Rikenella*-like bacteria microcolonies or it binds to a ligand to induce changes in the viability of *A. veronii* in the leech. These roles could explain why a mutation in the *rhs* gene significantly reduces the colonization ability of JG573 *in vivo* but not *in vitro*.

While this study has provided some insight into the role of the *A. veronii rhs* gene in the colonization of the leech digestive tract, further characterization of JG573 is necessary for a more complete understanding. In addition to the dose response curves to further characterize the *in vitro* phenotype of JG573, another future experiment is complementation of JG573 to the wild type *rhs* gene. If complementation restores the capability of JG573 to colonize the leech, this could be used to confirm that the decreased colonization ability is definitely due to the disruption in the *rhs* gene. By fully understanding the role that *rhs* protein plays in the colonization of the leech digestive tract, it may lead to better understanding of more complex systems, such as the human, in the future.

VI. CONCLUSIONS

1. The disrupted hypothetical protein in mutant JG573 is *rhs* family protein.
2. The data suggests that the wild type is more sensitive to certain antimicrobial compounds than JG573.
3. An important physiological change occurs inside the leech digestive tract between 18 h and 24 h after feeding that affects the viability of JG573.

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